# The podocyte's response to injury: Role in proteinuria and glomerulosclerosis

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**The terminally differentiated podocyte, also called glomerular visceral epithelial cell, are highly specialized cells. They function as a critical size and charge barrier to prevent proteinuria. Podocytes are injured in diabetic and non-diabetic renal diseases. The clinical signature of podocyte injury is proteinuria, with or without loss of renal function owing to glomerulosclerosis. There is an exciting and expanding literature showing that hereditary, congenital, or acquired abnormalities in the molecular anatomy of podocytes leads to proteinuria, and at times, glomerulosclerosis. The change in podocyte shape, called effacement, is not simply a passive process following injury, but is owing to a complex interplay of proteins that comprise the molecular anatomy of the different protein domains of podocytes. These will be discussed in this review. Recent studies have also highlighted that a reduction in podocyte number directly causes proteinuria and glomerulosclerosis. This is owing to several factors, including the relative inability for these cells to proliferate, detachment, and apoptosis. The mechanisms of these events are being elucidated, and are discussed in this review. It is the hope that by delineating the events following injury to podocytes, therapies might be developed to reduce the burden of proteinuric renal diseases.**

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Diabetic and non-diabetic glomerular diseases remain the major cause of chronic and end-stage renal disease. What makes the glomerulus fascinating, yet clinically challenging, is that there are four resident cell types that are potentially injured in different disease states. These include mesangial, endothelial, visceral epithelial (also called podocytes), and parietal epithelial cells. We and others classify glomerular disease based on which resident glomerular cell type is injured, as this provides a better understanding of why patients present clinically with nephritic and/or nephrotic syndromes. Diseases of mesangial cells (such as immunoglobulin (Ig)A nephropathy, lupus nephritis) and endothelial cells (such as thrombotic microangiopathy, lupus nephritis, mesangioproliferative glomerulonephritis, and others) typically cause nephritic syndrome. The parietal epithelial cell is a significant component of crescents in most forms of crescentic glomerulonephritis. In contrast, diseases of podocytes typically present with proteinuria, with or without nephrotic syndrome (Table 1). It should be noted that not all cases of nephrotic range proteinuria are owing to podocyte diseases, because the glomerular filtration barrier also comprises the glomerular endothelial cell (GEN) and glomerular basement membrane (GBM). Damage to these glomerular structures may therefore also present with nephrotic-range proteinuria, such as anti-GBM disease or thrombotic microangiopathy, respectively.

The focus of this forum is on podocytes, specifically how they respond to injury or damage, and how these events lead proteinuria and glomerulosclerosis.

## Normal podocyte structure

***Gross structure.*** Podocytes are highly specialized, terminally differentiated epithelial cells, with a quiescent phenotype.[1] Podocytes derive embryonically from mesenchymal cells.[2] Each mature podocyte has distinct anatomical, and therefore functional, components.[3] The cell body is at the center of the cell, and essentially lies in the urinary space. Herein lies the cell's nucleus, Golgi apparatus, and other cell machinery such as endoplasmic reticulum and mitochondria. From the cell body arise long primary processes, the ends of which contains foot processes. Foot processes in turn attach to the underlying GBM via integrins[4] and dystroglycans,[5] thereby anchoring this cell to the glomerular tuft.

**Table 1 | Diseases of the podocyte**

| Podocyte disease | Cause of injury | Mechanism/mediator |
|---|---|---|
| Membranous nephropathy | Anti-podocyte antibodies | C5b-9 |
| Minimal change disease | T cell mediated | Not well defined |
| Classic FSGS | Hereditary | α-Actinin-4 mutation<br>Podocin mutation<br>CD2AP haploinsufficiency |
| | Increased Pgc owing to:<br>• Obesity<br>• Diabetes<br>• Hypertension<br>• Reduced nephron number | Podocyte stress–tension |
| | ↓Podocyte number | Apoptosis<br>Detachment<br>Lack of proliferation<br>DNA damage<br>Hypertrophy |
| | Circulating factors | Permeability factor(s) |
| | Sporadic disease | α-Actinin-4 mutation<br>Podocin mutation |
| Cellular/collapsing FSGS | Infections | HIV<br>Parvo B19? |
| | Drugs | Pamidronate<br>Interferon |
| Diabetic nephropathy | Metabolic | Hyperglycemia |
| | Increased Pgc | Podocyte stress–tension |
| Amyloid | Amyloid protein deposition | Amyloid spicules directly injure podocyte |
| MPGN | Deposition of antigen–antibody complexes | Splitting of GBM<br>Podocyte effacement |

FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane.

Foot processes from neighboring podocytes overlap (interdigitate). The 'filtration slit' formed between adjacent interdigitating podocyte foot processes is a highly specialized gap junction called the slit diaphragm, which forms the major size barrier to protein leakage (see later for more details in slit diaphragm proteins).

***Molecular structure.*** Podocytes are polarized cells. Their unique shape is owing to an abundantly rich actin cytoskeleton, which serves as the podocyte's 'backbone'.[6] The actin cytoskeleton also enables podocytes to continually and dynamically alter shape, and also serves as a static function. The cytoskeleton comprises three distinct ultrastructural elements: (i) microfilaments (7–9 nm diameter), intermediate filaments (10 nm), and microtubules (24 nm). Microfilaments are the predominant cytoskeletal constituents of the foot process, and contain a dense network of F-actin and myosin. As will be discussed later, there are several actin-binding proteins such as synaptopodin[7] and α-actinin-4[8] in podocytes, which are important in maintaining podocyte shape.

The actin cytoskeleton is linked with other proteins. Kerjaschki[9] has classified podocytes into apical, basal, and junctional cell membrane domains, based on the molecular anatomy at each site. The *junctional domain* of proteins comprises those proteins comprising slit diaphragm proteins. Tryggvason was the first to discover nephrin,[10] a member of the Ig superfamily, as one of the now increasing number of complex slit diaphragm proteins. The cytoplasmic tail of nephrin binds to podocin.[11–13] Nephrin also interacts with and localizes to CD2AP.[14,15] More recently, another Ig superfamily of proteins have been identified called Neph-1, which interacts with nephrin, podocin, and FAT1.[16,17] Other slit diaphragm proteins include ZO-1, Neph-2 and -3, and densin.[18] By forming the only connection between adjacent podocytes, the slit diaphragm limits protein leakage by acting as a size barrier, analogous to a sieve. One is also left speculating that the slit may also function as a charge barrier, as some of these proteins are phosphorylated. As will be discussed later, certain slit diaphragm proteins actively participate in podocyte signaling, thereby enabling the slit to communicate with other podocyte proteins such as the actin cytoskeleton.

The *apical membrane domain* of podocytes is negatively charged, owing to the presence of the surface anionic proteins podocalyxin,[19] podoplanin,[20] and podoendin. This serves two functions. First, negative charge limits the passage of albumin (also negatively charged). Second, adjacent podocytes maintain separation by anion charge. The *basal domain* is required to anchor podocyte to the underlying GBM. $\alpha 3\beta 1$ integrin[21] and α- and β-dystroglycans[22] serve this function, and connect the body of the podocyte to certain matrix proteins within the GBM.

### Podocyte function

The complex architecture of constitutive proteins is required for the highly specialized functions of podocytes, which includes (i) a size barrier to protein; (ii) charge barrier to protein; (iii) maintenance of the capillary loop shape; (iv) counteracting the intraglomerular pressure; (v) synthesis and maintenance of the GBM; (vi) production and secretion of vascular endothelial growth factor (VEGF) required for GEN integrity. Therefore, it comes as little surprise that perturbations in one or more of these functions following podocyte injury underlies the signature clinical findings including marked proteinuria, typically nephrotic range, and often a decrease in renal function with elevated creatinine, both of which will be discussed in detail below.

## CAUSES OF PODOCYTE INJURY

From a clinical prospective, the predominant causes of nephrotic range proteinuria in adults owing to podocyte damage include focal segmental glomerulosclerosis (FSGS), membranous nephropathy, minimal change disease, membranoproliferative glomerulonephritis, amyloid, and diabetic nephropathy. However, for the most part, these diseases are named according to histologic descriptions of each disease, and do not inform one of the causes and mechanisms of each disease entity. The causes of podocyte injury in each disease entity are shown in Table 1, and each will be described briefly.

The classification of podocyte injury preferred by the author is to classify podocyte diseases into congenital, hereditary, and acquired causes. The latter is further divided into immune and non-immune causes. *Congenital* causes include abnormalities in structural podocyte proteins, and this is best exemplified by congenital nephrotic syndrome of the Finnish type. In this disorder, there are several different mutations in nephrin leading to a loss of normal podocyte function, resulting in the onset of fetal proteinuria.[23,24] Recent studies have shown that another congenital cause of podocyte damage is the development of maternal antibodies to neutral endopeptidase[25] and metallomembrane endopeptidase[26] in mothers who are deficient in the enzyme. As the fetus has the neutral endopeptidase antigen and the mother does not, the mother develops antibodies to this antigen, which cross the materno–fetal circulation, and deposit in podocytes, giving rise to membranous nephropathy. Ronco and Debiec[25] have recently shown a role for anti-neutral endopeptidase antibodies in certain cases of childhood onset membranous nephropathy. These antibodies are acquired *in utero*, and thus can be considered congenital. One of nephrin's binding partners, CD2AP gives rise to proteinuria in patients who have CD2AP haploinsufficiency.[27]

There are several *hereditary* causes of podocyte injury and proteinuria, and these typically include mutations in podocyte-specific proteins, of which mutations in α-actinin-4 and podocin are best defined. Pollak and co-workers identified that mutations in the podocyte actin-associated protein, α-actinin-4, causes autosomal-dominant FSGS.[8] Proteinuria typically develops in adulthood. Antignac and co-workers were the first to report that mutations in the slit diaphragm protein podocin causes autosomal-recessive steroid-resistant nephrotic syndrome and FSGS in children.[28] More recently, mutations in TRPC6, a newly discovered slit diaphragm protein, also leads to hereditary proteinuria.[29,30]

The majority of podocyte diseases are *acquired*, and these can be considered immune and non-immune mediated. The characteristic immune-mediated forms of podocyte injury are membranous nephropathy and minimal change disease, although one might also consider membranoproliferative glomerulonephritis associated with cryoglobulins as immune-mediated podocyte injury. The antibodies that cause 'idiopathic' membranous nephropathy remain elusive in man. Kerjaschki and Farquhar identified the Heymann nephritis antigenic complex, now called megalin, in rats as the auto-antigenic target.[31] Minimal change disease is considered immune-mediated because it is likely owing to an abnormality in T cells, although the precise mechanisms are not well defined.

Non-immune causes of acquired podocyte injury are multiple. These include infectious causes such as HIV-associated nephropathy giving rise to the characteristic collapsing glomerulopathy owing to the local infection of podocytes by the HIV virus.[32] Many speculate that Parvo B19 virus may also induce collapsing glomerulopathy in HIV-negative patients. The prototypical metabolic cause of podocyte injury is diabetes. Although diabetic nephropathy has long been considered a mesangial disease, it is also associated with significant podocyte injury (and hence marked proteinuria).[33] There is an increasing body of literature showing that stress–tension, a result of increased intraglomerular pressure, causes podocyte injury.[34,35] This is likely one of the final common pathways in systemic hypertension, diabetic nephropathy, the metabolic syndrome, and any cause of a reduced nephron number such as reflux nephropathy, or chronic glomerulopathies. Infiltrative diseases of podocytes are not common, and include amyloid, where studies have shown that individual amyloid spicules 'project' through the GBM, penetrating into the overlying podocytes. Finally, although the vast majority of slit diaphragm protein mutations are congenital or hereditary, recent studies have shown that sporadic FSGS can arise owing to mutations in podocin.[36] This leads one to ask if these patients have a 'two-hit' injury, that is, a gene mutation that by itself may not be sufficient to cause proteinuria, but in the presence of a second injury, such as hypertension or hypercholesterolemia, podocyte injury ensues.

## HISTOLOGIC CHANGES IN PODOCYTES FOLLOWING INJURY

Although serological and other laboratory tests are informative in glomerular diseases, the definitive diagnosis in most nephrotic syndromes is a renal biopsy. However, the range of abnormalities seen on pathological examination of the renal biopsy can be highly variable in diseases of podocytes. At one extreme, despite massive proteinuria, light microscopy can be

normal, such as minimal change disease. The other extreme is exemplified by classic or cellular FSGS, where glomerulosclerosis and glomerular tuft collapse are marked on light microscopy, with or without changes in podocyte number (increase or decrease).

Regardless of the cause of podocyte damage, typical podocyte abnormalities are best seen on electron microscopy and include vacuolization, microcystic, or pseudocystic changes, the presence of cytoplasmic inclusion bodies, and detachment from the GBM. In areas of reduced podocyte number, there may be focal areas of denudation of the underlying GBM. Although these changes are common, the characteristic response to podocyte damage/injury is a change in shape called effacement, and this will be discussed in detail below. It should be noted that these electron microscopy changes do not typically distinguish one podocyte disease from another, but rather represent a common final pathway of the podocyte's response to injury.

## HOW DOES PODOCYTE INJURY CAUSE PROTEINURIA AND GLOMERULOSCLEROSIS?

The clinical signature of podocyte damage is proteinuria, and in many instances, reduced renal function. The level of proteinuria can range from mild ($<3$ g/day) to nephrotic range ($>3$ g/day). The author will now focus the discussion on the mechanisms underlying proteinuria in response to podocyte injury (see Figure 1, Tables 2 and 3).

## PODOCYTE EFFACEMENT

### What is podocyte foot process effacement?

In 1957, Farquhar *et al.*[37] was the first to describe extensive foot process effacement in biopsies of patients with nephritic syndrome. Most authorities believe that effacement, also often referred to as fusion, retraction, or simplification, is a stereotypical reaction of podocytes to injury or damage. Scanning electron microscopy has shown that the change in podocyte shape called effacement consists of gradual simplification of the inter-digitating foot process pattern, resulting in the formation of a cell that looks flat and elongated. This is not fusion of neighboring cells. Rather, it is owing to retraction, widening, and shortening of the processes of each podocyte. The frequency of filtration slits is reduced,[38] giving the appearance of a continuous cytoplasmic sheet covering the GBM. Effacement is not specific to one disease, but rather is synonymous with podocyte injury of many forms.

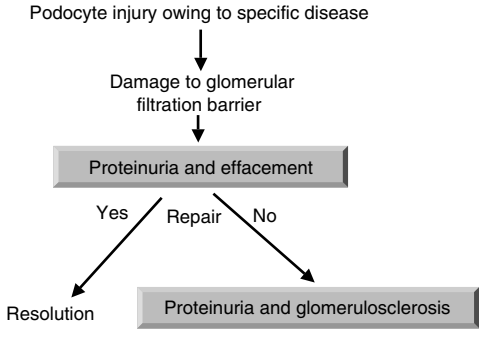


**Figure 1 | Podocyte response to injury.** Podocytes are injured by immune- and non-immune-mediated disease, resulting in damage to the glomerular filtration barrier. This typically results in proteinuria and effacement. The fate of the podocyte then depends on several factors, such as reparative mechanisms. If these are present, and/or the initial injury is halted, there may be resolution. However, if injury persists, and/or there are inadequate repair mechanisms presents, proteinuria persists, with the development of glomerulosclerosis, leading to reduced renal function.

Animal models showed that effacement starts as a decrease in the degree of interdigitation by shortening and widening of foot processes. This is accompanied by degradation of some foot processes, followed by loss of the inter-digitating foot process pattern between individual cells. Foot process length decreases up to 70%, and the width increases up to 60% compared to normal. The resultant abnormal cell shape comprising a flattened and spread out cell is what we know as effacement. Studies have shown that effacement is not simply a passive phenomenon, but rather is an active process that is energy dependent, and is initiated by changes in the podocyte's cytoskeleton.

### Podocyte effacement and proteinuria: What is the chicken and what is the egg?

The author does not know the answer to this seemingly easy question, and believes that there may be three views, and that

**Table 2 | Mechanisms leading to proteinuria following podocyte injury**

| Cause of proteinuria following podocyte injury | Specific podocyte defect |
|---|---|
| Slit diaphragm proteins | Nephrin mutation in man |
| | Podocin mutation in man |
| | CD2AP haploinsufficiency in man |
| | FAT-1-targeted deletion in mice |
| | Neph-1-targeted deletion in mice |
| Reduced podocyte number | Detachment |
| | Apoptosis |
| | Lack of adequate proliferation |
| | DNA damage |
| | Hypertrophy |
| Podocyte effacement | Changes in slit diaphragm proteins |
| | Abnormal podocyte–GBM interaction ($\alpha$,$\beta$ dystroglycans, $\alpha3\beta1$ integrin) |
| | Actin cytoskeleton reorganization owing to synaptopodin, $\alpha$-actinin-4, CDK5 |
| | Loss of negative charge |
| | Injury to apical membrane proteins (podocalyxin, NHERF2, Ezrin) |
| Loss of podocyte anion charge | ↓Podocalyxin |
| | ↓GLEPP |
| Abnormal GBM | Proteases from podocyte |
| | Oxidants from podocyte |
| | GBM thickening owing to matrix accumulation from podocyte |
| | ↓Heparan sulfate proteoglycan |
| Glomerular endothelial cell dysfunction | ↓VEGF from podocyte |

CDK, cyclin-dependent kinase; GBM, glomerular basement membrane; GLEPP, glomerular epithelial protein; NHERF, $Na^+/H^+$-exchanger regulatory factor; VEGF, vascular endothelial growth factor.

**Table 3 | Mediators and effects following podocyte injury**

| Mediator from podocyte | Effect |
|---|---|
| Reactive oxygen species | Creation of holes in GBM |
| | Podocyte apoptosis |
| | Podocyte DNA damage |
| | Effacement |
| | Lipid peroxidation |
| | Protein peroxidation |
| Angiotensin II | Podocyte apoptosis |
| | Podocyte hypertrophy |
| | Increases TGF-$\beta$ levels $\rightarrow$ matrix accumulation |
| | Increases VEGF levels $\rightarrow$ matrix accumulation |
| | Reduces nephrin levels |
| | Increases p27 levels |
| | Increases IP-8 and IP-10 levels |
| Metalloproteinases | Alteration in GBM matrix |
| | Disruption of nephrin–NEPH2 complex |
| Prostaglandins | Endoplasmic reticulum stress |
| Mechanical stretch | Podocyte detachment |
| | Podocyte apoptosis |
| | Inhibition of podocyte proliferation |
| | Podocyte hypertrophy |
| TGF-$\beta$ | Increase in matrix proteins leading to GBM thickening |
| | Podocyte apoptosis |
| | Increases metalloproteinases |
| SPARC | Podocyte detachment |
| cAMP | ?Narrowing of filtration slits |
| | ?Podocyte relaxation |
| VEGF | $\uparrow$TGF-$\beta$ levels |
| | $\uparrow$Production of $\alpha$3(IV) collagen by podocytes |
| Signaling pathways | ERK, JNK, SAPK |

ERK, extracellular signal-regulated kinase; GBM, glomerular basement membrane; JNK, c-Jun $NH_2$-terminal kinase; NEPH, nephrin-neutral endopeptidase; SAPK, signal-activated protein kinase; SPARC, secreted protein acid rich in cysteine; TGF-$\beta$, transforming growth factor-$\beta$; VEGF, vascular endothelial growth factor.

the truth lies somewhere in between these. The *first* view is that effacement itself is sufficient to cause proteinuria. There are numerous examples where podocyte effacement is accompanied by proteinuria in experimental models and human disease. Indeed, one is usually hard pressed to find marked proteinuria without effacement in human disease. Data shows that indeed effacement precedes proteinuria in experimental models such as puromycin aminonucleoside nephrosis.[39] Effacement results in a decrease in the filtration slit frequency along the GBM and has been associated with narrowing of the filtration slits and development of actual tight junctions between foot processes.[40] Effacement also causes apical displacement of the slit diaphragm. These data, while association only, lead one to speculate that once a podocyte undergoes effacement from a specific cause, it is sufficient to cause proteinuria.

The *second* view is that effacement is the final common pathway to one of several abnormalities in, or injuries to, podocytes, and therefore is not the cause of proteinuria. Thus, in this scenario, effacement itself does not cause proteinuria, but is rather a 'marker' for the actual cause of proteinuria (see below for causes of effacement). This view suggests that podocyte effacement is not required for proteinuria, and that one can have significant podocyte abnormalities in the absence of effacement. Support for this notion comes from Chugh and co-workers, who induced proteinuria by disrupting the Neph1—nephrin complex with specific antibodies, and showed proteinuria with preserved foot processes.[41] Salant's group also showed proteinuria in rats following the administration of the anti-5-1-6 (nephrin) antibody, in the absence of effacement.[42]

The *third* school of thought is that effacement can occur independent of proteinuria, and that foot process effusion is not a necessary consequence of proteinuria.[43] For example, a recent study by Lahdenkari[44,45] showed that effacement can occur in the absence of proteinuria. Van den Berg and co-workers studied humans with minimal change nephrotic syndrome and showed significant differences in the degree of effacement in patients with minimal change nephrotic syndrome, membranous nephropathy, and IgA, and that the differences were independent of the level of proteinuria.[46] Rather, the authors showed that it was the widths and shape of the slit diaphragms that correlated with proteinuria. Foot process width was 580 nm, whereas untreated minimal change nephrotic syndrome patients had a mean foot process width of 1600 nm. Further studies are clearly needed to verify these interesting observations, and to provide increased molecular understanding of these events.

Of interest is how effacement might explain the decrease in glomerular filtration rate in certain nephrotic states. It is estimated that the filtrations slits provide for 50% of the hydraulic resistance of the glomerular capillary wall. Recall that the ultrafiltration coefficient (Kf) is determined by the total length of the filtration slit between foot processes. The latter is decreased by foot process effacement. Thus, the decrease in total slit length and number of slit diaphragms owing to effacement may explain the decreased hydraulic permeability of the filtration barrier as well as decreased glomerular filtration rate in certain nephrotic diseases such as minimal change disease.[45]

### Actin cytoskeleton: the backbone of podocyte shape

As stated earlier, one of the major functions of podocytes is to provide structural support to the glomerular tuft. Foot processes are highly dynamic in large part owing to a rich actin cytoskeleton.[47,48] Microtubules and vimentin-type intermediate filaments are distributed in the cell body and primary processes. In the major processes, the cytoskeleton is composed mainly of microtubules, interwoven with intermediate filament proteins. Microtubule-associated protein-2, -3, and -4, as well as Tau protein have been described in podocytes. In contrast to the cytoskeletal proteins in the cell body and major processes, foot processes have an elaborate microfilamant-based contractile apparatus composed of actin, myosin-II, $\alpha$-actinin, talin, and vinculin. As will be discussed below, these anchor to entire foot process to the underlying GBM via integrins.

The actin cytoskeleton ultimately determines the podocyte's shape. Proteins regulating or stabilizing the actin cytoskeleton are therefore critical in the normal function of the podocyte, and any alterations in the actin itself, or in actin-regulating proteins, might lead to changes in podocyte shape, and therefore function. Ichimura *et al.*[48] demonstrated that within adult podocytes, the actin cytoskeleton is divided into two populations: (i) actin bundles that run along the longitudinal axis of foot processes above the level of the slit diaphragm, and (ii) a cortical actin network that distributes beneath the plasma membrane of the foot process. Foot process effacement is usually associated with an increase in the microfilaments.

Dontscho Kerjaschki[9] described three cell membrane domains (apical, basal, and slit diaphragm) of podocytes in his articulate review. Each of these domains, and the actin-associated proteins, synaptopodin and α actinin-4, ultimately connect to, and therefore have the potential to regulate, the actin cytoskeleton. The effect of each membrane domain and the actin-associated protein will now be discussed below in the context of the development of effacement as a response to podocyte injury.

## CAUSES OF PODOCYTE EFFACEMENT

### Actin-associated proteins

The major podocyte actin-binding proteins are α-actinin and synaptopodin (Table 4).

***Alpha actinin-4.*** α-Actinin-4 is an actin filament cross-linking protein, and thus co-localizes with actin in podocytes. Podocyte foot process effacement is preceded by an increase in α-actinin-4 levels in experimental proteinuric disease.[49] α-Actinin-4 mutations in man is associated with proteinuria and effacement,[8] and either knocking out or overexpressing α-actinin-4 in mice leads to proteinuria and effacement.[50,51]

### Synaptopodin

Synaptopodin is a novel class of actin-associated proteins, without homology to any other class of proteins, and was discovered by Peter Mundel.[7] It is expressed in podocytes, and in telencephalic dendrites. Synaptopodin is a proline-rich linear protein and contains two high score PEST (a sequence rich in proline (P), glutomic acid (E), serine (S) or threonine (T)) sites, which target proteins for rapid degradation. However, until a recent landmark paper by Mundel and co-workers, its function has been an enigma.[52] Mundel has recently shown that there are three synaptopodin isoforms in podocytes, named synaptopodin-long, -short and -T. Mundel showed that in podocytes, synaptopodin interacts directly with α-actinin-4. Moreover, synaptopodin modulates the expression of α-actinin, by elongating α-actinin-induced actin filaments.

The function of synaptopodin has been delineated utilizing null mice.[52] Administering protamine sulfate to mice causes podocyte effacement. The recovery is normal in wild-type mice given heparin, which neutralizes protamine sulfate. In contrast, recovery from effacement is abnormal in synpatopodin-null mice given protamine sulfate then heparin. Taken together, synpatopodin has a critical role in maintaining normal podocyte shape, and likely does this by altering α-actinin-4 function.

**Table 4 | Causes of podocyte effacement**

| Mechanism underlying effacement | Mediator of effect |
|---|---|
| Changes in slit diaphragm proteins | Nephrin |
| | Podocin |
| | FAT-1 |
| | CD2AP |
| | Neph1 |
| Abnormal podocyte–GBM interaction | Integrins |
| | Integrin-linked kinase |
| | SPARC |
| Actin cytoskeleton reorganization | Rho GTPases |
| | α-Actinin-4 |
| | Synaptopodin |
| | CDK5 |
| Changes in negative charge | Podocalyxin |
| | GLEPP |

CDK, cyclin-dependent kinase; SPARC, secreted protein acid rich in cysteine.

### Slit diaphragm proteins and podocyte effacement

The discovery of nephrin by Karl Trygvvason was an incredible milestone in our understanding of what prevents proteins from traversing the slit diaphragm. There are several reviews that the reader is encouraged to read for a more in-depth discussion on individual slit diaphragm proteins, as these are beyond the scope of this discussion.

The inter-digitiating foot processes form a 40 nm wide filtration slit. Two adjacent podocytes form a continous membrane-like structure that we call the slit diaphragm, which is the size barrier to proteins. Studies have shown that nephrin, a transmembrane adhesion protein of the Ig superfamily encoded by *NPHS1* gene, is one of the major proteins constituting the slit diaphragm reviewed by Tryggvason.[53] Mutations in the genes encoding proteins for nephrin results in proteinuria. A second protein, podocin, encoded by the gene called *NPHS2*, has been shown to bind to nephrin. Podocin-null mice or patients with podocin mutations develop proteinuria, and patients typically have steroid-resistant nephrotic syndrome.[11,28] The multi-adapter protein CD2AP cloned by Shaw has been shown to also interact with nephrin at the slit diaphragm.[15] CD2AP-null mice die of massive proteinuria, and CD2AP haploinsufficiency leads to glomerular disease in humans.[27] Other slit diaphragm proteins including Neph1 and FAT1 also complex with nephrin, and targeted deletions in mice lead to proteinuria.[16,41,54,55] It is clear that the slit acts like a sieve, in that it is the significant size barrier to protein.

Of note, in mice and man, mutations/abnormalities in the slit diaphragm proteins listed above all display foot process effacement. Administration of anti-Neph1 and anti-nephrin result in rapid disorganization of the actin cytoskeleton, leading to podocyte effacement.[41,42] This raises the question how might a slit diaphragm protein regulate podocyte shape,

which as discussed earlier, is ultimately owing to changes in the actin cytoskeleton. Elegant studies by Benzing and Walz have shown that slit diaphragm proteins are not static, but rather are constantly involved in signaling processes.[17] Thus, slit diaphragm proteins send signals regulating the podocyte's polarity, survival, and cytoskeleton organization. Several studies have elucidated how slit diaphragm proteins interact with proteins 'outside' of the slit diaphragm to regulate normal podocyte shape and therefore effacement following injury. CD2AP connects the nephrin complex with the actin-modifying proteins WASP, CAPZ, cortactin, and the Arp2/3 complex. The protein ZO-1 directly associates with the cortical actin cytoskeleton, and Densin binds to α-actinin-4.[18] FAT-1 is also an organizer of actin polymerization. Taken together, there is an increasing body of literature showing that the actin cytoskeleton is regulated in part through signaling pathways from specific slit diaphragm proteins.

Thus, when slit diaphragm proteins are injured/mutated, in addition to loss of the size barrier, there is also loss of actin arrangement (i.e. cytoskeleton disorganization), leading to podocyte effacement and proteinuria. Salant's group showed the fraction of membrane-associated nephrin that is bound to actin is diminished in experimental membranous nephropathy, changes that correspond to alterations in podocyte morphology.[56] Taken together, several studies favor the notion that proteinuria associated with effacement is not owing to effacement *per se*, but is rather owing to increased protein leak at the level of the slit diaphragm, and that effacement is a consequence of slit diaphragm protein damage.

### Changes in podocyte–GBM interaction

The basal cell membrane domain of the podocyte has also been shown to have a role in maintaining cell shape. Two important constituents include the $\alpha 3\beta 1$ integrin and α and β dystroglycans, which serve as matrix receptors for podocytes, and thus serve to tether podocytes to the GBM (reviewed by Kretzler[57]).

*α- and β-Dystroglycans* are normally localized in a linear pattern outlining the contours of the capillary loops in the 'soles' of podocytes.[5] The α chain contains a polyanionic binding site for the cationic laminin globular-binding domain common to several matrix proteins such as laminin, agrin, perlecan, and proteoglycans. The non-covalently attached β chain links the dystroglycan complex to the cortical actin cytoskeleton. The levels of dystroglycan are reduced in minimal change disease, and are redistributed in a clustered manner in FSGS, diseases associated with podocyte effacement. Proof of principle was provided by Kerjaschki and co-workers, who showed that injury to podocytes with reactive oxygen species or protamine sulfate directly splits the attachments of dystroglycans, and this leads to the induction of podocyte effacement.[58]

*Integrins* are heterodimeric transmembrane molecules mediating cell–matrix interactions. The major podocyte integrin is $\alpha 3\beta 1$, which binds collagen, fibronectin, laminin, and entactin/nidogen in the GBM.[59] Blocking $\beta 1$ intergins with an antibody induces foot process fusion and effacement, as well as podocyte detachment. Kreidberg *et al.*[21] used a genetic approach, and showed that in the $\beta 1$-null mouse, podocytes are effaced. These mice die at birth. *Integrin-linked kinase* is involved in cell signaling and Kretzler's group[60] has shown that abnormalities in this lead to podocyte effacement and proteinuria.

### Cell cycle proteins and podocyte shape

We recently showed that a novel cell-cycle-protein, called cyclin-dependent kinase 5 (CDK5), is constitutively expressed in normal mature podocytes *in vitro* and *in vivo*.[61] CDK5-null mice are embryonically lethal owing to neurological defects. In order to explore the role of CDK5, Griffin *et al.*[61] reduced CDK5 levels in cultured podocytes with either small interfering RNA or a specific inhibitor, Roscovitine. In both circumstances, reducing CDK5 levels or activity resulted in a marked change in podocyte shape. Interestingly, unlike other cyclin-dependent kinases, CDK5 has no role in podocyte proliferation.

### Changes in the apical membrane proteins

In addition to preventing proteinuria by acting as a size barrier, podocytes, like the GBM on which it sits, also act as a negative charge barrier to prevent the passage of anionic proteins. This is owing to the constitutive presence of the anionic apical proteins called podocalyxin and podoplanin.

*Podocalyxin* is a heavily sialyated and sulfated membrane protein belonging to the family of sialomucins. Neutralization of the anionic surface charge with protamine sulfate or by removal of sialic acid leads to foot process flattening, and rearrangement of the cell junction between adjacent podocytes.[62] The levels of podocalyxin are virtually suppressed by exposing cultured podocytes to high glucose conditions, and podocalyxin levels were also reduced in the streptozotocin rat model of experimental diabetic nephropathy.[63] Podocalyxin overexpression inhibits cell–cell adhesion, and likely serves to maintain an open filtration pathway between neighboring foot processes, and neutralizing the charge affects cell–cell adhesion and junctional permeability. These studies have recently been extended by Farquhar, who showed recently that the cytoplasmic domain of podocalyxin is linked to the actin cytoskeleton through $Na^+/H^+$-exchanger regulatory factor (NHERF)/ezrin complexes,[64] and that podocalyxin is required to maintain foot process structure *in vivo*. Taken together, podocalyxin functions to maintain podocyte shape by linking to the actin cytoskeleton, and a decrease in levels or loss of anionic charge leads to podocyte shape changes (effacement) and distortion of the slit diaphragm, both leading to proteinuria.

*Podoplanin* is a 43 kDa glycoprotein.[65] Levels are reduced in the puromycin aminonucleoside nephrosis model. Studies have shown that injecting rats with an anti-podoplanin antibody results in podocyte effacement, and proteinuria.

## CHANGES IN PODOCYTE NUMBER: THE LINK TO GLOMERULOSCLEROSIS

### Evidence and relevance for reduced podocyte number in disease

There is an increasing body of experimental and clinical literature showing a decrease in podocyte number in diabetic and non-diabetic glomerular disease (Table 5).[66–69] Wiggins showed that aging is also associated with reduced podocyte number. The consequences of reduced podocyte number include proteinuria and glomerulosclerosis. The mechanism underlying proteinuria is simply owing to a lack of charge and size selectivity in areas of podocyte loss, as these barriers are now devoid. Studies have shown that proteinuria increases as podocyte number decreases.[33,67,68]

Recent studies have also correlated podocyte loss with the onset and magnitude of glomerulosclerosis. The reader is referred to excellent reviews by Kriz for the explanation how reduced podocyte number leads to glomerulosclerosis, as this is beyond the scope of the current discussion.[66,70,71] In brief, podocyte loss leads to areas of 'bare or denuded' GBM where podocytes are reduced. Because one of the functions of podocytes is to maintain capillary loop shape by opposing the outward forces of glomerular pressures (which are increased in many renal diseases), podocyte loss leads to outward bulging of the GBM in the denuded areas. A synchial attachment forms upon contact of the denuded GBM with the parietal epithelial cells and Bowmans capsule. This is the first 'committed step' for the formation of FSGS.

Podocyte number reflects the balance between processes that favor an increase in cell number, and those that favor a decrease in cell number. In most, but not all forms of progressive glomerular disease, the factors favoring a decrease in podocyte number prevail, and hence overall number decreases. We see this clinically as proteinuria and increasing creatinine. The causes of reduced podocyte number are discussed below (Figure 2).

### Podocyte detachment: hanging on needing to stay attached

Podocytes are normally tethered to the underlying GBM by integrins and dystroglycans, and are essentially 'floating' in the urinary space. It has been known for some time that podocytes and certain podocyte-specific protein products can be detected in the urine from patients with proteinuric renal disease, but are typically absent in healthy subjects, and in non-podocyte glomerular diseases.[72–77] More recent studies have shown the presence of intact podocytes in the urine in experimental and clinical studies of diabetic and non-diabetic glomerular diseases. Interestingly, Lemley and Petermann[78–81] showed that many of the podocytes present in the urine in disease are indeed viable. Pichler recently showed that high glucose levels itself is sufficient to cause detachment of cultured podocytes (personal communication). Yu *et al.*[81] have recently shown that perhaps the presence of podocytes in the urine is a more sensitive marker than proteinuria. Taken together, these studies show that podocytes are present in the urine in certain diseased states.

The mechanisms of podocyte detachment have not been well delineated. Recall that podocytes attach to the GBM by adhering to underlying matrix GBM proteins via intergins and dystroglycans. Thus, an obvious hypothesis is that alterations in the levels or activities of these 'adherence molecules' favors detachment of podocytes from the GBM. Indeed, studies have shown a decrease in $\alpha 3\beta 1$ integrin in diabetes,[82] and it is therefore tempting to speculate that this might underlie podocyte detachment in diabetic nephropathy. Finally, as will be discussed below, once podocytes detach from the GBM, they are also extremely vulnerable to undergoing apoptosis.

### Podocyte apoptosis: a life or death decision

A second cause underlying a decrease in podocyte number is increased programmed cell death, also called apoptosis. There is an emerging experimental and clinical literature showing that apoptosis is a major cause of reduced podocyte number, leading to proteinuria and/or glomerulosclerosis. Bottinger was among the first to show increased podocyte apoptosis in experiments performed in tumor growth factor-$\beta$ (TGF-$\beta$) transgenic mice.[83] Wiggins and co-workers showed podocyte apoptosis in the puromycin aminonucleoside nephrosis model of podocyte injury, and that glomerulosclerosis was only first detected following a 20% reduction in podocyte number.[84] Moreover, there was a direct correlation thereafter between reduced podocyte number and increased sclerosis.

What are the causes of podocyte apoptosis? A simple view is that the decision whether a cell undergoes apoptosis or

**Table 5 | Podocyte number in glomerular diseases**

| Podocyte number normal | Podocyte number decreased | Podocyte number increased |
|---|---|---|
| Membranous nephropathy | Membranous nephropathy | HIV-associated nephropathy |
| Minimal change disease | Diabetic nephropathy | Cellular/collapsing FSGS |
| Classic FSGS | Classic FSGS | Crescentic glomerulonephrtitis |
| | Amyloid | |
| | Aging | |

FSGS, focal segmental glomerulosclerosis.

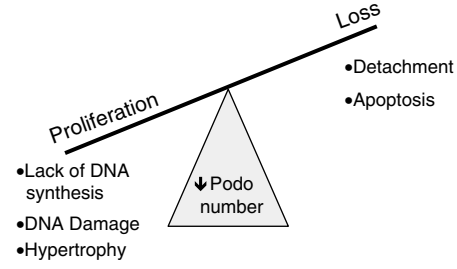


**Figure 2 | Factors governning podocyte number.** Total podocyte (podo) number is a balance between proliferation and loss. Podocyte number is reduced by either a decrease in proliferation owing to lack of DNA synthesis, DNA damage or hypertrophy, and/or an in crease in podocyte loss owing to detachment and apoptosis.

survival and injury is that this fate is ultimately the balance between pro- and antiapoptotic (i.e. survival) factors. These are discussed below (Table 6).

***Survival mechanisms in podocytes.*** What prevents podocytes from dying, and what keeps podocytes alive following injury? One obvious response to this question is that proteins or molecules that are constitutively present in a cell are essential for normal function, such as survival. This raises several candidates. Accordingly, recent studies have focused on the possibility that specific slit diaphragm proteins govern podocyte survival. Clues emerged from CD2AP-null mice, where Schiffer *et al.*[89] showed that podocyte apoptosis was increased significantly in these mice compared to CD2AP –wild-type mice. A role for CD2AP in protecting podocytes from death was further validated when the proapoptotic cytokine TGF-$\beta$ increased apoptosis in cultured podocytes derived from CD2AP-null mice compared to control. These authors, and Benzing's group[86] showed that CD2AP selectively engages the phosphatidylinositol 3′-kinase kinase/AKT antiapoptotic signaling pathway. When CD2AP is absent or likely reduced, active (phosphorylated) AKT is dramatically reduced, and this is associated with increased susceptibility to podocyte apoptosis.

Recent data from Benzing has also shown a role for nephrin in reducing podocyte apoptosis.[86] Nephrin too increases Akt activity, and thereby reduces podocyte apoptosis. Saleem showed that podocytes mutant for nephrin have increased apoptosis in response to serum starvation compared to wild-type cells.[98] This effect was related to nephrin phosphorylation. Saleem showed that VEGF reduces apoptosis in podocytes expressing nephrin, but that this survival effect was absent in nephrin mutant cells. The mechanism for this effect is that VEGF-induces phosphorylation of nephrin. Taken together, nephrin is required for podocyte survival, and by phosphorylating nephrin, VEGF secreted by podocytes acts as an autocrine survival factor, as perhaps does insulin-like growth factor-1.[95] Griffin from our group recently showed that cyclin I, which is constitutively expressed in normal podocytes, functions as a survival factor.

Podocyte attachment is a critical survival mechanism. As stated earlier, once podocytes detach from the GBM, their susceptibility and propensity to undergo apoptosis increase significantly. However, the mechanisms for this remain unclear. Thus, although no published studies have yet shown a direct role, one can speculate that $\alpha3\beta1$ integrin and the dystroglycan complex are required for podocyte survival by facilitating adhesion to the GBM. What happens to podocytes if the constituents of the GBM upon which podocytes sit are altered. Cybulsky's group showed that cultured podocytes grown on collagen have increased survival, an effect that was mediated through the activation of focal adhesion kinase and the Ras–ERK signaling pathway.[93] These studies give rise to the notion that perhaps in diseases where the matrix proteins of the GBM are altered following podocyte injury, such as membranous and diabetic nephropathy, podocyte survival is reduced. Finally, Wada *et al.*[91] from our group has shown a role for Bcl-2 in protecting podocytes from apoptosis.

More recently, Wada showed that dexamethasone markedly reduces podocyte apoptosis in cultured podocytes in response to puromycin aminonucleoside and TGF-$\beta$.[91] This novel non-immunological effect is owing to a decrease in p53 levels by dexamethasone. More studies are needed to fully understand the mechanisms involved in the protective actions of dexamethasone on podocyte apoptosis.

***Proapoptotic factors in podocytes.*** The cytokine TGF-$\beta$ and its receptors increase in a variety of podocyte diseases, including membranous nephropathy, diabetic nephropathy, and FSGS. In addition to its profibrotic effects, there is now an emerging literature showing that TGF-$\beta$ also induces podocyte apoptosis. Schiffer *et al.*[83] have delineated some of the downstream mediators of TGF-$\beta$-induced podocyte apoptosis. They showed that SMAD-7 augments TGF-$\beta$-induced apoptosis, and that p38 mitogen-activated protein kinase and caspase-3 are required for TGF-$\beta$ to induce podocyte apoptosis.

Our group has focused on delineating the nuclear events in TGF-$\beta$-induced podocyte apoptosis, and focused on the CDK inhibitor p21. The rationale for this is that like TGF-$\beta$, p21 is increased in podocytes in experimental membranous nephropathy (PHN model),[99] diabetic nephropathy (Streptozotocin model),[100] and minimal change disease (PAN model). Wada *et al.*[88] showed that TGF-$\beta$ increases p21 levels in cultured podocytes. To determine if p21 was required for the apoptotic effects of TGF-$\beta$, p21-null podocytes in cultured were studied. The results showed that TGF-$\beta$ only induced apoptosis in wild-type podocytes where p21 was present (and increased by TGF-$\beta$), but not in p21-null podocytes. However, reconstituting p21 expression in p21-null podocytes by transfection restored the apoptotic response to TGF-$\beta$. Taken together, in addition to certain signaling events, the CDK inhibitor p21 is necessary for TGF-$\beta$-induced apoptosis.

**Table 6 | Regulation of podocyte life and death**

| Proapoptotic | Antiapoptotic (prosurvival) |
|---|---|
| Angiotensin II[34,85] | Cyclin I |
| $AT_1$ receptor[34,85] | Nephrin[86,87] |
| TGF-$\beta$ [83,88] | CD2AP[89,86] |
| Cyclosporine[90] | Dexamethasone[91] |
| SMAD 7[83] | Bcl-2[91] |
| Reactive oxygen species | Cell–cell contact |
| Detachment | VEGF[87] |
| $\downarrow$p21[92] | Collagen via Ras-ERK signaling[93] |
| $\downarrow$p27[94] | Focal adhesion kinase[93] |
| Hyperglycemia | Insulin-like growth factor-1[95] |
| Stress–tension[96] | Hepatocyte growth factor[90] |
| bFGF[97] | |
| Lytic concentrations of C5b-9 | |
| p53[91] | |
| Apoptotic-inducing factor[91] | |

bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase; TGF-$\beta$, transforming growth factor-$\beta$; VEGF, vascular endothelial growth factor.

There is a large clinical literature showing that inhibiting angiotensin II and/or its receptor with a angiotensin-converting enzyme inhibitor and angiotensin receptor blocker, respectively, improves proteinuria, and that this benefit is independent of blood pressure lowering. These studies suggested that angiotensin II has deleterious effects directly on the kidney, independent of its hemodynamic actions. Indeed, Singhall showed that angiotensin II directly causes podocyte apoptosis, an effect that is mediated through the AT1 receptor.[85] Durvasula *et al.*[34] showed that when cultured podocytes are placed under stress–tension (induced by mechanical stretch), they apoptose. However, blocking the AT1 receptor pharmacologically significantly reduced stress–tension-induced podocyte apoptosis. Taken together, in addition to reducing systemic and intraglomerular pressures, angiotensin II blockade likely also reduces podocyte apoptosis, thereby minimizing podocyte loss, providing an additional mechanism explaining how these agents reduce proteinuria and glomerulosclerosis.

It has been well established that diabetes is associated with reduced podocyte number, and that this correlates with the onset and magnitude of proteinuria.[33] We and others[29,30] have shown that hyperglycemia directly induces apoptosis in cultured podocytes, thereby providing an additional possible explanation to reduced podocyte number in this disease.

Puromycin aminonucleoside is a podocyte toxin, and induces apoptosis.[101] The mechanisms of this effect are being clarified. We recently showed that this effect was mediated by the tumor suppressor gene product, p53, because inhibiting p53 prevented apoptosis.[91] In addition, puromycin aminonucleoside also increases the levels of the proapoptotic protein, Bax. Thus, the pathways underlying podocyte apoptosis are becoming clearer.

Finally, many cells are more vulnerable to apoptosis upon cell cycle entry. Our data show that podocyte apoptosis is increased in rats with the PHN model of membranous nephropathy following administration of basic fibroblast growth factor, coinciding with increased cell cycle entry.[99] Moreover, apoptosis is also increased when podocytes proliferate in the absence of the CDK inhibitors p21[92] and p27.[94]

The studies discussed above show that there are now several well-established pro- and antiapoptotic mechanisms at play in podocytes, and that following injury, the balance of these effects determines the cell's fate to live or die. The latter seems to predominate in instances of progressive glomerulosclerosis, and suggests that future interventions should be aimed at preventing or reducing podocyte apoptosis.

### Lack of adequate podocyte proliferation

A third cause underlying reduced podocyte number is the apparent lack of proliferation in response to injury.[102] Podocytes are terminally differentiated epithelial cells, and as such, seem to attempt to maintain this quiescent phenotype at all costs, even at the expense of developing glomerulosclerosis. Why then do podocytes not proliferate in certain diseases such as membranous nephropathy, minimal change disease, diabetic nephropathy, and classic FSGS, yet proliferate in others such as collapsing/cellular FSGS and crescentic glomerulonephritis? To answer this fundamental question, we have focused our efforts on the role of specific cell cycle regulatory proteins. The rationale for this approach is that a cell's growth phenotype is closely governed by its state of differentiation, and that both these events may share common regulatory control at the level of the cell cycle. Studies have shown that the state of differentiation and proliferation are indeed very closely linked in podocytes. During glomerulogenesis, presumptive and immature undifferentiated podocytes have a proliferative phenotype.[2] In contrast, mature adult podocytes have a quiescent phenotype. Quiescent cells are in the $G_0$ phase of the cell cycle (i.e. have excited cell cycle). In order for differentiated cells such as podocytes to proliferate requires that they de-differentiate and then re-enter cell cycle at $G_1$ phase. This is followed by DNA synthesis in the S phase, and mitosis occurs in the M phase. Cell division (called cytokinesis), which follows mitosis, is strictly speaking not part of the cell cycle.

Based on the relationship between podocyte differentiation and proliferation, we consider diseases of podocytes as those that remain differentiated and quiescent (minimal change disease classic FSGS, membranous nephropathy, diabetes), and those that de-differentiate and proliferate (cellular/collapsing FSGS, crescentic glomerulonephritis). I will now discuss what governs the decision of these cell fates at the level of the cell cycle. In order for a cell to transition through the cell cycle, specific cyclins must bind to and activate partner CDKs at each phase of the cell cycle.[103,104] However, certain CDK inhibitors limit proliferation by inhibiting target cyclin–CDK complexes.

***Cyclins and CDK's in podocytes.*** Cyclin D1 increases following podocyte injury, as does the protein levels of cyclin A and its partner CDK2 in the passive Heymann nephritis model of membranous nephropathy.[99,105] Thus, podocytes undergo DNA synthesis, *albeit* at very low levels. This is in stark contrast from mesangial cells, where cyclin A–CDK2 activity is markedly increased in mesangial proliferative glomerulonephritis.[106] These data show that the lack of podocyte proliferation in the majority of podocyte diseases is not owing to the lack of the cell cycle machinery (i.e. cyclins and CDKs) required to proliferate. More recently, studies have shown that podocytes do proliferate in crescentic glomerulonephritis.[107] Our data show that CDK2 activity is markedly increased in experimental crescentic glomerulonephritis.[108] Inhibiting CDK2 activity with Roscovitine significantly reduces podocyte proliferation in this model, and improves renal function. CDK2 inhibition also significantly improves renal function in experimental HIV-associated nephropathy[109] and experimental membranous nephropathy.[110]

Why then does CDK2 activity and hence DNA synthesis increase in some (collapsing/cellular FSGS and crescentic glomerulonephritis), but not other (classic FSGS, membra-

nous, and diabetes) podocyte diseases. One possibility is that the catalytic partners for CDKs are not increased. There is little support for this because cyclins D1, A, and B increase in podocytes following injury.[111] The more likely explanation is that despite an increase in cyclin–CDK levels, there is a marked increase in CDK inhibitors, which reduce/inhibit cyclin–CDK activity, and this will now be discussed.

***CDK inhibitors in podocytes.*** Although there are two families of CDK-inhibitors, little is known about the role of the inhibitors of CDK4 (INK) family (p15, 16, 18, 19) in podocytes. The discussion will therefore focus on the role of the CDK-interacting protein/kinase inhibitor protein (CIP/KIP) family of CDK inhibitors (p21, p27, p57). In the normal human and rodent glomerulus, staining for p21 is absent, but there is a constitutive expression of p27 and p57.[112] Within the glomerulus, p57 staining is restricted to podocytes.[112]

***p21.*** There is a marked increase in p21 levels in podocytes in experimental membranous nephropathy (PHN model), coinciding with the lack of proliferation.[99] Moreover, p21 was shown to bind to and inhibit cyclin A–CDK2 in this model. To test the role for p21 in governing podocyte proliferation, we induced injury in p21-wild-type and p21-null mice. Podocyte proliferation was markedly increased in nephritic p21-null mice compared to nephritic p21–wild-type mice, and this was associated with worse renal function.[92] These results are consistent with the notion that p21 limits podocyte proliferation following injury.

***p27.*** In cultured immortalized podocytes, the switch from a proliferative and undifferentiated phenotype to a non-proliferative and quiescent phenotype coincides with an increase in p27 protein levels.[113] In the PHN model of membranous nephropathy, p27 levels increase in podocytes.[99] By extracting protein from isolated glomeruli, we showed that p27 also has increased affinity for cyclin A–CDK2 complexes. To test the role of p27 in governing podocyte proliferation, experimental injury was induced in wild-type and null mice.[94]The onset and magnitude of glomerular proliferation was markedly increased in nephritic p27 null, and the majority of the cells proliferating were podocytes. This was associated with increased accumulation of matrix proteins, and a decline in renal function compared to nephritic wild-type mice. In human disease, p27 levels do not decrease in non-proliferative podocyte diseases such as classic FSGS, minimal change, and membranous.[112] In contrast, p27 levels decrease in collapsing FSGS, and this corresponds to the increase in podocyte proliferation in this disease. Taken together, p27 is a critical regulator of podocyte proliferation following injury.

***p57.*** We are particularly interested in p57 because expression is limited to podocytes under normal conditions.[113] p57 levels do increase coinciding with immortalized podocytes become quiescent in culture.[113] p57 levels decrease in proliferating podocytes *in vivo* in experimental glomerular disease. Like p27, p57 levels decrease in areas of podocyte proliferation in human collapsing FSGS, whereas p57 levels remain unchanged in non-proliferative human podocyte disease.[112]p57-null mice are embryonically lethal, making the study of these mice currently invalid to test the precise role of this CDK inhibitor. Taken together, the author speculates that p57 may not be required to maintain podocyte quiescence under normal conditions, but is required to limit proliferation following injury.

## Podocyte proliferation

The conventional thought has been that podocytes do not proliferate. However, using genetically manipulated mice that express green-colored podocytes, Holzman and co-workers showed that podocytes proliferate and increase in number in experimental crescentic glomerulonephritis.[107] Recent studies also support the notion that podocytes proliferate in collapsing/cellular FSGS, although this has been more difficult to prove.[114] In experimental HIV-associated nephropathy, Klotman's group showed that the NEF antigen of the virus is sufficient to induce podocyte proliferation.[115] This is owing to a decrease in the CDK inhibitor p27, and an increase in cyclins D1 and Nelson *et al.*[109] showed that reducing podocyte proliferation with a CDK2 inhibitor improved overall renal survival and reduced proteinuria.

Taken together, a change in podocyte number, whether a decrease (as occurs in most glomerular diseases) or an increase, is detrimental to normal glomerular function. The mechanisms underlying reduced and increased podocyte number will be discussed in further detail.

## DNA damage

Although we have reported that one mechanism underlying the apparent lack of adequate proliferation was an abnormality in DNA synthesis, we have also recently shown that another possibility is an abnormality in mitosis owing to a block at the $G_2$/M checkpoint of the cell cycle. When quiescent cultured mesangial cells and podocytes are exposed to antibody and a complement source to induce sublytic injury (defined as less than 5% lactate dehydrogenase release), both cell types enter the cell cycle at $G_1$ phase.[116,117] In mesangial cells, this is followed by robust DNA synthesis, and completion of the cell cycle, with resultant proliferation and increased cell number.[118] In contrast, podocytes undergo DNA synthesis, albeit limited, but do not proliferate because they arrest at the $G_2$/M phase of cell cycle.[117] In seeking to determine the mechanisms underlying this difference, we showed that sublytic C5b-9 injury causes DNA damage in podocytes, but not in mesangial cells.[119] DNA damage prevents proliferation by arresting cells at $G_2$/M phase. In podocytes, DNA damage was accompanied by an increase in p53, p21, GADD45, and checkpoint kinase-1 and -2.[119]These proteins coordinate to halt the cell cycle at the $G_2$/M checkpoint, thereby arresting cells before mitosis. More recently, Marshall from our group showed that DNA damage also occurs in podocytes in response to other forms of injuries, such as puromycin aminonucleoside, and that this is mediated in part by reactive oxygen species. As shown in

Table 3 oxidants therefore reduce podocyte number by at least two mechanisms, apoptosis and DNA damage.

### Hypertrophy

An interesting response to podocyte injury is an increase in cell size owing to hypertrophy.[120–122] This is defined biochemically as an increase in the cell's protein to DNA ratio, accompanied by increased cell volume. This requires that the cell enters the cell cycle in $G_1$, which leads to protein synthesis, and thus an increase in protein content. However, cells arrest at the $G_1$/S checkpoint, thereby preventing an increase in DNA content, which would have occurred at S phase. These events lead to an increased cellular protein:DNA ratio, and increased cell size (but not number). There are several studies that have shown podocyte hypertrophy *in vivo*, including in diabetic nephropathy and FSGS.

Why do podocyte's hypertrophy? The belief is that podocyte hypertrophy is initially adaptive, and that this is an attempt by a cell that is relatively incapable of proliferating, to cover the underlying GBM and thus reduce proteinuria in denuded areas where neighboring cells have detached or apoptosed. However, with time, it is likely that podocyte hypertrophy becomes maladaptive. Cell culture studies have shown potential mechanisms underlying this observation. First, high glucose is sufficient to cause podocyte hypertrophy *in vitro*.[123] This might be mediated by an increase in angiotensin II, which itself, can cause hypertrophy. Angiotensin II-mediated podocyte hypertrophy might be regulated through an increase in the CDK inhibitor p27, which is increased in podocytes *in vitro* and *in vivo* in diabetes. Nagata and co-workers showed that podocyte hypertrophy may be owing to increased expression of specific CDK inhibitors, p21 and p27.[124]

More recently, Petermann *et al.*[125] from our group showed that injuring cultured podocytes with mechanical stretch inhibited proliferation, and switched the cell to a hypertrophic phenotype. This was mediated by the CDK inhibitor p21.

These studies show that despite entry into cell cycle, under certain circumstance, podocyte's arrest at the $G_1$/S checkpoint, and undergo a hypertrophic growth response. These events therefore limit the podocyte's ability to proliferate, leading to a reduction in overall podocyte number.

## PODOCYTE INJURY LEADS TO ABNORMALITIES IN THE GBM

### Matrix accumulation

Studies have shown that the GBM serves as a charge, and perhaps size barrier to proteins. It is therefore of little surprise that injury to the GBM, such as occurs in anti-GBM disease and Alport's syndrome, may result in proteinuria. As discussed earlier, podocytes are closely adherent to the GBM, begging the question that following podocyte injury, is the GBM impacted in any way. Several excellent reviews describe the normal matrix protein makeup of the GBM, which is beyond the scope of this discussion.[126] Recall that the GBM itself is acellular, and thus cannot make its own matrix proteins. In states where the GBM is thickened, such as membranous and diabetic nephropathies, this is owing to an increase in the accumulation of 'new' and usually 'abnormal' matrix proteins, which are produced by the overlying injured podocytes. One can therefore speculate that the change in composition of the GBM in these states of podocyte injury disturb the normal GBM architecture, and thus function, leading to proteinuria directly, or as described earlier, indirectly by inducing podocyte apoptosis.

### Oxidants

In several types of podocyte injury *in vitro*[127] and in experimental diseases such as membranous nephropathy (PHN model), diabetic nephropathy, minimal change disease (PAN model),[128] and other models,[129] injured or damaged podocytes increase the production and release of oxidants.[130] Oxidants in turn cause damage directly to podocytes, including the induction of apoptosis, DNA damage, and likely lipid and protein oxidative stress (Table 3).[131,132] However, oxidants also damage the underlying GBM, by creating holes directly in the GBM. The latter leads to a reduction in size barrier, and results in proteinuria. Oxidants likely also induce proteinuria by other mechanisms. Oxidants induce foot process effacement, and administering antioxidants to rats with PAN normalizes podocyte shape, and reduces proteinuria in this model, and in experimental membranous nephropathy.[130] Finally, oxidants also disrupt $\alpha$3-integrins in experimental proteinuric renal disease, leading to proteinuria.[133]

### Proteinases

During glomerulogenesis, podocytes are required for the synthesis of several extracellular matrix proteins that constitute the GBM. Many believe that podocytes are also required for the 'maintenance' of the GBM in the mature glomerulus. The quantity of matrix proteins in any cell or membrane is a balance between synthesis and degradation. Metalloproteinases are proteinases specific for basement membrane proteins, and serve to reduce the abundance of these proteins such as type IV collagen, fibronectin, and laminin. Cathepsin L, metalloproteinase-2, and metalloproteinase-9 are proteinases endogenous to podocytes, the activities of which are increased by cytokines such as TGF-$\beta$.[134] Metalloproteinase-9 is markedly increased in podocytes following C5b-9 injury in experimental membranous nephropathy.[135] More recently, proteinases have been shown to induce proteinuria by also interfering with nephrin-neutral endopeptidase H interaction in the slit diaphragm.[136]

These studies show that the increase and release of oxidants and proteinases by podocytes following injury may alter the matrix constituents of the underlying GBM, rendering it more leaky to protein. The author proposes that a vicious podocyte–GBM–podocyte cycle then develops, where the altered matrix proteins in the GBM now may lead to deleterious effects on podocytes, such as apoptosis and detachment.

## PODOCYTE INJURY LEADS TO ENDOTHELIAL CELL DYSFUNCTION

The third component of the glomerular filtration barrier is the GEN, which is a highly specialized fenestrated cell. There is an interesting 'cross-talk' between podocytes and GENs that is served by VEGF. GEN themselves do not make VEGF, but have VEGF receptors. Earlier studies showed that VEGF expression is reduced in diabetic nephropathy, but the consequences of this were speculated upon. Elegant genetics studies by Quaggin has carefully elucidated precisely how podocyte-secreted VEGF regulates GEN development and function.[137] Utilizing a nephrin-cre-LoxP mouse model system to delete selectively podocyte VEGF-A, Quaggin showed that loss of one VEGF-A allele causes glomerular endotheliosis. Deleting both VEGF-A alleles prevents the formation of the glomerular filtration barrier. Interestingly, overexpressing VEGF-A causes collapsing glomerulopathy. Taken together, a paradigm is emerging whereby a decrease in podocyte secreted VEGF leads to abnormal GEN function, which in turn may lead to proteinuria. The role of VEGF as a podocyte survival factor was discussed earlier. Thus, another mechanism whereby podocyte injury leads to proteinuria is by affecting GEN.

## NEW THOUGHTS ABOUT OLD PODOCYTE THERAPIES, AND NEW POSSIBILITIES ON THE HORIZON

I will end my discussion with a few thoughts on how old therapies alter podocyte biology and thus proteinuria, and briefly discuss potential new therapies.

### Angiotensin blockade

One of the mainstays of treating proteinuria is angiotensin II blockade. However, given that the proteinuric-reducing effects are independent of blood pressure lowering suggests that angiotensin II likely has local effects on podocytes directly (Figure 3). Indeed, angiotensin II is increased in podocytes following exposure to mechanical stretch and hyperglycemia. Angiotensin II causes podocyte apoptosis,[34,85] actin rearrangement, increased VEGF synthesis,[138] increased TGF-$\beta$ levels,[138] reduction of ZO-1 and nephrin levels leading to proteinuria,[139] and alterations in calcium and cAMP signaling. Overexpressing the AT1 receptor in podocytes causes marked glomerulosclerosis.[140] Thus, inhibiting angiotensin II production or its receptor with an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, respectively, mitigates many deleterious effects on podocytes, thereby reducing the response to injury.

### Corticosteroids

Corticosteroids are the mainstay of therapy for several proteinuric diseases. In addition to its immune-modulating effects, recent studies have shown that corticosteroids may have direct effects on podocytes. Wada *et al.*[91] from our group recently showed that dexamethasone reduced podocyte apoptosis induced by puromycin aminonucleoside and TGF-$\beta$. Dexamethasone prevented the increase in the proapoptotic tumor suppressor gene, p53. Wada also showed that dexamethasone altered the subcellular localization of apoptotic inducing factor. Taken together, these studies show that corticosteroids may reduce proteinuria and/

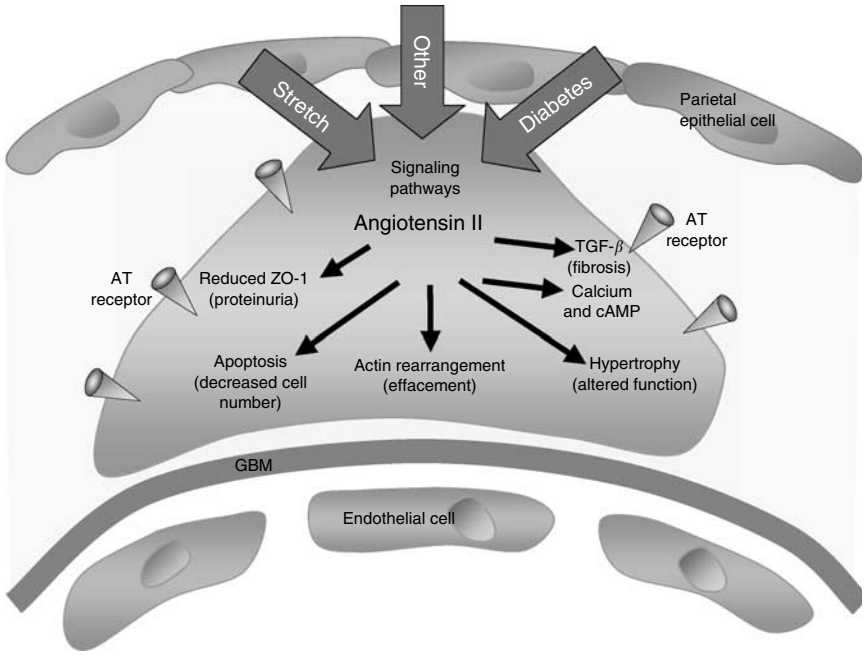


**Figure 3 | Injurious effects of angiotensin II on podocytes.** The numerous deleterious actions of angiotensin II (AT) and its receptor are shown, providing the rationale for the use of angiotensin-converting enzyme Inhibitors and angiotensin receptor blockers in patients with proteinuria.

glomerulosclerosis by preventing a decrease in podocyte number. Ransom *et al.*[141] recently showed that dexamethasone increases ciliary neurotrophic factor, αB-crystallin and heat shock 27 in podocytes, which are viewed as kidney protective proteins. The same group also showed that dexamethasone enhanced actin stability against subsequent disruption by cytochalsin D and latrunculin A. Thus, corticosteroids may directly improve proteinuria and effacement through a variety of factors, and also reduce glomerulosclerosis by reducing podocyte apoptosis.

### All-*trans*-retinoic acid

Podocytes express receptors for all-*trans*-retinoic acid (ATRA). Several recent studies have shown a role for ATRA in diabetic and non-diabetic proteinuric diseases).[142] For example, Vaughan from our group showed that giving ATRA to rats with experimental podocyte disease significantly improved proteinuria, and that this was likely owing to the prevention of decreasing nephrin and podocin expression.[143] We also have shown that ATRA reduces glomerulosclerosis in HIV transgenic mice (M Fleet, unpublished data). The protective effects of ATRA on podocytes need to be further delineated.

### CDK2 inhibitors

Several small molecule inhibitors have been developed in the past decade, which target specific proteins, such as CDK2. We recently administered the CDK2 inhibitor Roscovitine to mice with experimental anti-GBM disease characterized by podocyte proliferation.[108] Our data showed that Roscovitine improved proteinuria and blood urea nitrogen compared to animals receiving vehicle. This was associated with a decrease in podocyte proliferation. Nelson *et al.*[109] recently showed marked improvement in renal function in HIV transgenic mice given the CDK2 inhibitor cyclacel, and Floege's group showed improvement in renal function when cyclacel was given to rats with experimental membranous nephropathy (PHN model).[110] Taken together, there may be future potential for the use of small molecule inhibitors in proteinuric states owing to podocyte disease.

## CONCLUSIONS AND FUTURE DIRECTION

In many glomerular diseases, the exact mechanisms causing the disease are not yet fully delineated. However, our understanding of the response to podocyte injury has increased significantly in the past decade following the explosion of molecular and cellular research in podocyte biology. The response of podocytes to injury is complex, and involves numerous processes. Many of these overlap in several podocyte diseases, yet some are specific. The author believes that we are in an exciting era where newer diagnostic approaches, treatment guidelines, and prognostic indicators based on molecular endpoints are on the horizon. It is the hope that newer and specific therapies will also be developed that alter the deleterious responses to podocyte injury, thereby reducing the burden of renal disease. With the exciting ongoing research efforts worldwide, there is hope for our patients.

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